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# The Role of Calcium Ions in Toxic Cell Injury

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**Calcium ions have been increasingly implicated as a mediator of the mechanisms generating lethal cell injury under a variety of pathologic circumstances. An overview of the various roles suggested for such alterations in cellular calcium homeostasis is presented.**

**The central role of plasma membrane damage in the genesis of irreversible cell injury is used to divide the postulated roles for calcium ions into two major mechanisms. On the one hand, calcium ions have been proposed as mediators of the functional consequences of plasma membrane injury. An influx of extracellular calcium ions across a damaged permeability barrier and down a steep concentration gradient may convert potentially reversible injury into irreversible injury. On the other hand, alterations in intracellular calcium homeostasis are postulated to participate in the mechanisms generating potentially lethal plasma membrane injury. The release of calcium stores sequestered within intracellular organelles raises the cytosolic concentration of free calcium, a process that may activate, in turn, a number of membrane-disruptive processes. The data supporting these two distinct actions of calcium are reviewed and discussed.**

## Introduction

In recent years there has been some considerable interest in the role that calcium ions may play in the pathogenesis of toxic cell injury. The present report gives a brief overview of the current status of the relationship between toxic cell death and alterations in intracellular calcium homeostasis.

All cells in the body exist in quite profound disequilibrium with their external environment. This state is maintained both actively and passively by the plasma membrane of the cell. It is now generally accepted that any damage to the plasma membrane disrupting the maintenance of this disequilibrium between the internal and external environments may ultimately result in the death of a cell. In turn, alterations in calcium homeostasis have been implicated as a mediator of the mechanism leading to lethal plasma membrane damage and the functional consequences of this injury transforming a living cell into a necrotic one.

## Calcium Ions as the Mediator of the Consequences of Plasma Membrane Damage

Calcium has the largest gradient of any chemical across the plasma membrane of all living cells. The concentration of calcium ions in extracellular fluids is in the millimolar range ($10^{-3}$ M). By contrast, the calcium ion concentration in the cytosol is some 10,000-fold lower on the order of $10^{-7}$ M. This large concentration gradient is maintained by both the passive impermeability of the plasma membrane to calcium ions and by the active extrusion of calcium from the cell.

Cell death is almost invariably accompanied by a number of morphologic changes that are recognizable by the naked eye and by light microscopy and are labeled coagulative necrosis. Given the large calcium ion gradient across the plasma membrane of all cells, it is not surprising that coagulative necrosis is accompanied by the accumulation of calcium ions. Calcium ions are biologically very active, and their accumulation in dead or dying cells may actually contribute to the morphologic transformations characterizing coagulative necrosis. Thus, the influx and accumulation of calcium ions and the resultant morphologic changes of coagulative necrosis can account for the common morphology of cell death, despite the ultimate cause, whether by a toxic chemical, a virus, ischemia, etc.

The sequence of events leading to coagulative necrosis may then be summarized as (*a*) irreversible injury and cell death, (*b*) loss of the plasma membrane's ability to maintain a gradient of calcium ions; (*c*) an influx and accumulation of calcium ions in the cell; and (*d*) the morphologic appearance of coagulative necrosis. Under such a scheme, coagulative necrosis occurs after the point of no return—that is, after irreversible injury and death of the cell.

Alternatively, cell injury may lead to potentially reversible plasma membrane damage. As a result of this damage, however, the large gradient of calcium

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ions can no longer be maintained. Excess calcium ions then accumulate in the injured cells and cause coagulative necrosis. This alternative scheme has two specific implications. First, it does not define a stage of cell death distinct from coagulative necrosis. Second, it envisions the accumulation of calcium ions as the point at which potentially reversible cell injury becomes irreversible.

What is the evidence for such a role of calcium ions? It must be emphasized at the outset that this postulated role for an influx of calcium as a mechanism that converts potentially reversible to irreversible injury can only be studied in the intact animal. With tissue culture systems cell viability is generally assayed by using procedures that reflect plasma membrane damage—that is, by the entrance of substances normally excluded from viable cells, such as trypan blue; or by the release of materials normally confined to the cells, such as intracellular enzymes. In other words, coagulative necrosis is not the endpoint for the assessment of cell death *in vitro*. Thus, it is not really possible to consider the postulated role of calcium ions in the pathogenesis of coagulative necrosis with cell culture models. This point is not widely appreciated and needs to be stressed.

Clearly, it is possible to disrupt the plasma membrane of cells in culture such that the cells will be scored as dead without a requirement either for the presence extracellular calcium ions or for their entry into the cells. Alternatively, it is possible to manipulate the culture conditions in a manner to produce a minimal damage to the plasma membrane. This in turn allows sufficient calcium ions to enter the cell, initiating secondary events that further disrupt the plasmalemma and result in the cells being scored as dead. We need to emphasize that neither such calcium-independent or calcium-dependent cell death *in vitro* bears on the pathogenesis of coagulative necrosis in the intact animal.

Unfortunately, there have been very few studies that have used intact animal models to specifically address the role of calcium ions in toxic cell death. The study of galactosamine hepatitis provided some support for the hypothesis linking an influx of calcium ions to irreversible liver cell injury. Within two hours of the administration of galactosamine to an intact rat, the liver cells evidenced changes in their plasma membranes and slight increases in the total liver cell calcium content (*1*). However, the liver cells were not necrotic at this point. The liver cell calcium content continued to rise between 2 and 8 hr with the appearance of necrotic cells, and the calcium content did not return to normal until some 24 to 36 hr later. Chlorpromazine given 2 hr after the galactosamine prevented any further rise in total liver cell $Ca^{2+}$ content for at least 24 hr. At the same time, chlorpromazine prevented the appearance of liver cell necrosis (*2*).

The interpretation of this experiment is dependent on the mechanism of action of chlorpromazine ions. Chlorpromazine has a number of effects on biologic membranes, particularly, inhibiting the flux of calcium ions across several different membranes (*3*). In turn, this action has been used to explain the ability of chlorpromazine inhibiting a number of physiologic phenomena thought to be dependent on an increased calcium ion flux (*4–9*). Chlorpromazine may be similarly acting on the galactosamine-intoxicated liver cells to block an influx of calcium ions across a damaged plasma membrane. Such a situation would argue for a role for the influx of calcium ions in the genesis of irreversible cell injury. However, it must be emphasized that there is no direct evidence that, in the galactosamine-intoxicated hepatocyte, chlorpromazine is deterring the development of liver cell necrosis by preventing an influx of calcium ions across a damaged plasma membrane.

Ischemic liver necrosis in the intact rat is another model in which there is some evidence implicating an influx of calcium ions in the genesis of irreversible cell injury. The reperfusion of a rat liver that has been ischemic *in situ* from 2 to 3 hr is accompanied by a rapid increase in the total calcium content of the liver and the development of liver cell necrosis (*10*). Administration of chlorpromazine just prior to the reestablishment of blood flow reduces the extent of the subsequent live cell necrosis (*10*). Again, such a result is consistent, but does not establish the point that an influx of calcium ions may be related to the transition from potentially reversible to irreversible cell injury. Clearly, much more work with intact animal models is needed before a critical judgment can be rendered as to the validity of the hypothesis implicating calcium ions in the pathogenesis of coagulative necrosis.

## Calcium Ions as the Mediator of Plasma Membrane Damage

In recent years there has been considerable interest in the role of alterations in intracellular calcium homeostasis as a mediator, rather than the consequence of the plasma membrane injury that can result in toxic cell death. Orrenius and coworkers (*11–18*) proposed that a disturbance in intracellular calcium homeostasis is an early event in the genesis of lethal injury produced by an acute oxidative stress. This hypothesis is based on experimental data of three sorts.

First, exposure of rat hepatocyte suspensions to *tert*-butyl hydroperoxide or hydrogen peroxide generated by the redox cycling of menadione impaired the sequestration of calcium by mitochondria and the endoplasmic reticulum. There was an accompanying rise in the cytosolic free $Ca^{2+}$ concentration, as assessed by the activity of glycogen phosphorylase *a* or by the use of fluorescent indicators. This disturbed calcium homeostasis was paralleled by a depletion of glutathione resulting from the reduction of either

peroxide by glutathione peroxidase and the oxidation of pyridine nucleotides accompanying the reduction of GSSG by glutathione reductase. Blebbing of the surface membrane followed these alterations in both calcium and glutathione metabolism.

Second, certain conditions, notably the presence of the sulfhydryl reagent dithiothreitol, that protected against cell injury by an oxidative stress, also prevented the increase in cytosolic calcium and the loss of $Ca^{2+}$ from its intracellular storage pools. The oxidation of GSH and NADPH and the blebbing of the surface membrane of the hepatocytes were similarly prevented by dithiothreitol.

Finally, direct mobilization of calcium from intracellular stores by the ionophore A23187 also promoted cell killing, preceded by a rise in cytosolic calcium. On the basis of these observations, a model of oxidative injury was proposed whereby the ultimate loss of viability of the liver cells is a consequence of a sequence of events proceeding from GSH depletion to an elevated cytosolic calcium concentration and finally to membrane injury.

However, these same data do not exclude alternative interpretations in which the rise in cytosolic calcium after an oxidative stress is not an essential factor in membrane damage, but rather a consequence thereof or an unrelated epiphenomenon. In the course of our studies of the mechanisms mediating cellular injury by hydrogen peroxide (*19–21*), we have developed various methods manipulating the sensitivity of hepatocytes to an oxidative stress. In particular, we have shown that toxicity of hydrogen peroxide or *tert*-butyl hydroperoxide is dependent on a cellular source of ferric iron (*21,22*). Cultured hepatocytes treated with the ferric iron chelator deferoxamine are resistant to the toxicity of $H_2O_2$ or *tert*-butyl hydroperoxide (*21–23*). However, deferoxamine did not prevent the rise in cytosolic calcium ion concentration occurring with the exposure of cultured hepatocytes to hydrogen peroxide generated either in the medium by glucose oxidase or intracellularly by the metabolism of menadione (*22*). Similarly, deferoxamine pretreatment did not prevent the rise in cytosolic calcium ion concentration accompanying the metabolism of *tert*-butyl hydroperoxide by cultured hepatocytes (*23*). Furthermore, sulfhydryl reagents inhibited the rise in cytosolic calcium ion concentration in deferoxamine-pretreated hepatocytes. In other words, the concentration of cytosolic calcium ions could be manipulated up or down without any parallel changes in the viability of the cells.

Conversely, cultured hepatocytes were depleted of calcium ions by treatment with EGTA in a calcium-free medium. Importantly, calcium-depleted hepatocytes were not resistant to the toxicity of hydrogen peroxide despite the virutal elimination of the rise in cytosolic calcium ion concentration (*23*).

These data show that the loss of liver cell viability from an acute oxidative stress can be dissociated from changes in intracellular calcium homeostasis. It is important to emphasize that this was achieved by two independent means. On the one hand, changes in calcium homeostasis were shown to occur without any accompanying irreversible liver cell injury. On the other hand, irreversible cell injury was achieved without changes in intracellular calcium homeostasis. These data suggest that the alteration in intracellular calcium homeostasis induced by an oxidative stress is an epiphenomenon that can be dissociated from the development of lethal cell injury in cultured hepatocytes.

This conclusion does not preclude the fact that an elevated cytosolic calcium ion concentration can initiate a sequence of biochemical events resulting in damage to the plasma membrane and, thus, death of suspended or cultured hepatocytes. It is possible to kill cultured hepatocytes by a mechanism that is clearly dependent on a disordered intracellular calcium homeostasis. The calcium ionophore A23187 can readily be shown to kill cultured hepatocytes only in the presence of extracellular calcium ions. Hepatocytes that were pretreated with A23187 were killed in a dose-dependent manner by the addition of calcium ions to the culture medium (*22*). There was a similar dose-dependent rise in cytosolic calcium ion concentration. Importantly, the cytosolic calcium ion concentration seemed to be higher than that which occurred with hydrogen peroxide at a comparable cell killing (*22*). Furthermore, deferoxamine pretreatment and sulfhydryl reagents had no effect on the loss of viability with this calcium-dependent cell killing.

This demonstration of calcium-dependent cell killing with the ionophore A23187 raises a persistent question. Are there other circumstances where a raised cytosolic calcium ion concentration initiates mechanisms that result in damage to the plasma membrane and eventually in cell death? Our studies emphasize that an acute oxidative stress is not necessarily such a circumstance. Furthermore, the widespread use of suspensions of isolated hepatocytes in an atmosphere of 95% $O_2$ may lead to somewhat misleading conclusions with respect to the role of alterations in intracellular calcium homeostasis in toxic cell injury. Such suspensions of freshly isolated hepatocytes are subject to at least two problems that do not seem to occur with cultured hepatocytes.

First, suspended hepatocyts may be more sensitive to the presence of extracellular calcium ions. One example will illustrate this problem: catechol-protected suspensions of hepatocytes from the toxicity of *tert*-butyl hydroperoxide (*24*). In this situation,while there is no effect on the accumulation of GSSG accompanying the metabolism of *tert*-butyl hydroperoxide by glutathione peroxidase, catechol did reduce the rise in the cytosolic calcium ion concentration accompanying the depletion of GSH (*24*). Researchers argued that catechol protected the hepatocytes by a mechanism related to lowering the cytosolic calcium ion concentration (*24*).

By contrast, the protective effect of catechol against the killing of cultured hepatocytes by *tert*-butyl hydroperoxide had no effect on the increase in cytosolic calcium ion concentraton (*23*). Such a discrepancy is readily explained by the antioxidant action of catechol and the use of suspended versus cultured hepatocytes. Freshly suspended hepatocytes exposed to an atmosphere of 95% $O_2$ and 5% $CO_2$ are more fragile than cells cultured for 24 hr with room air (20% $O_2$). Lipid peroxidation occurs in the cultured hepatocytes as rapidly as the rise in cytosolic calcium (*23*). We suspect that such lipid peroxidation in suspended hepatocytes renders them readily permeable to extracellular calcium ions, an effect that would not occur until later with the cultured cells. In other words, we are arguing that the level of cytosolic calcium detected in the suspended hepatocytes that were exposed to *tert*-butyl hydroperoxide in the presence of catechol (*24*) was the same as the level in the cultured hepatocytes that were protected by other mechanisms unrelated to the change in calcium homeostasis (*23*). Thus, caution must be exercised in drawing conclusions with regard to the lethal consequences of alterations in intracellular calcium homeostasis from the use of suspensions of freshly isolated hepatocytes.

An influx of extracellular calcium ions with suspended hepatocytes may aggravate the underlying mechanisms of irreversible cell injury and unnecessarily complicate the interpretation of the experiments. Such a conclusion is supported by the observation that removal of calcium ions from the medium reduced, but did not prevent, the extent of killing of cultured hepatocytes by *tert*-butyl hydroperoxide. This result documents that both calcium-independent and calcium-dependent events contribute to the cell killing.

There is a second difficulty with the use of suspensions of fresh hepatocytes. In the absence of extracellular calcium, such cells rapidly lose much of their endogenous antioxidant activity and become susceptible to oxidative stress (*25*). The addition of vitamin E or other exogenous antioxidants protects the cells. Such an oxidative stress does not occur in the presence of extracellular calcium nor in cultured hepatocytes maintained in the absence of extracellular calcium. Thus, it is difficult to manipulate suspensions of isolated hepatocytes like one can cultured hepatocytes in order to critically address the role of calcium ions in toxic cell injury.

In summary, it remains a challenge to investigative toxicology to define both the role of alterations in intracellular calcium homeostasis in the pathogenesis of coagulative necrosis, as well as the mechanisms mediating the damage to plasma membranes leading to this morphologic hallmark of cell death.

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